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14. ABSTRACT

We have proposed to develop tethered Hsp90 inhibitor capable of carrying various radioactive iodine isotopes for early detection and ablation of metastatic breast cancers. These probes specifically target a surface form of heat shock protein 90 (eHsp90)that is expressed on malignant cells and internalized. In this report we describe our chemistry efforts to synthesize a non-radioactive tethered Hsp90 inhibitor, methods developed for stannylation of the molecule such that it can be effectively radioiodinated with either I121 or I124. Two paths of synthesis were developed. A standard operating procedure was developed and transferred to our collaborator who developed protocols for radioiodination. An in active control molecule was also developed. These molecules will be tested as imaging agents in mouse models of breast cancer.

15. SUBJECT TERMS
Radiodination, tethered Hsp90 inhibitor, malignant breast tumor, ectopic Hsp90

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Table of Contents

Page

1. Introduction……. 3

2. Keywords……. 3

3. Accomplishments……..……... 4

4. Impact……...…… 11

5. Changes/Problems...…..…… 12

6. Products…….……..…..……. 13

7. Participants & Other Collaborating Organizations…… 13

8. Special Reporting Requirements…… 13

9. Appendices…… 13

1. Introduction

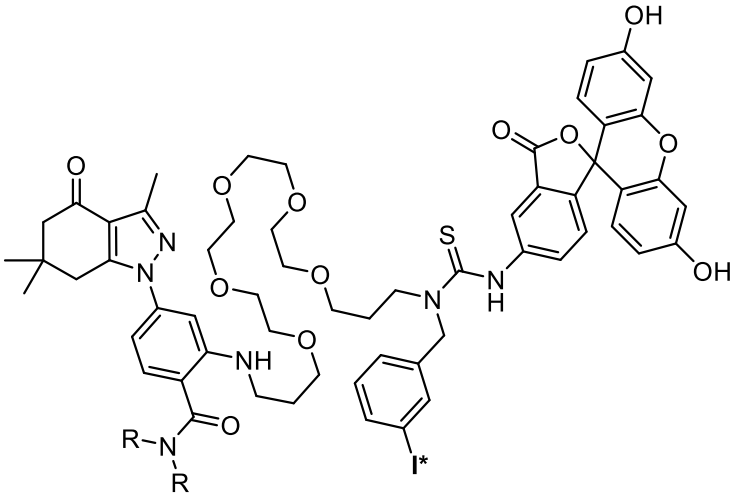
In the US, routine breast cancer screening results in over 1.6 million biopsies annually leading to the diagnosis and surgical resection of breast cancer or breast carcinoma *in situ* in over 250,000 women respectively. Unfortunately, the sensitivity but low specificity of screening has led to concerns about over treatment of indolent disease, as evidenced by the increased incidence and treatment of early stage breast cancer without a concomitant decrease in the nearly 40,000 breast cancer deaths annually. Clinical data indicate a strong link between high expression/activation of Heat shock protein 90 (Hsp90) with poor prognosis in malignant breast cancer (Cheng et al., 2012; Pick et al., 2007). Specifically, immunohistochemical analysis of breast cancer cell lines and 655 primary breast cancers (including 331 ER+ and 324 ER- tumors) showed increased Hsp90 expression in all breast cancer cell lines, and in nearly 90% of primary breast cancers (Pick et al., 2007). A recent study at our institution evaluated Hsp90 gene expression from profiles of over 4,000 breast cancer patients from 23 publically available gene expression databases, which also reported overall survival data from over 1000 patients. This study confirmed up regulated Hsp90 was associated with poor overall survival in all breast cancer subtypes including estrogen (ER) negative, HER2 negative and triple negative breast cancers (Cheng et al., 2012). Our laboratories recently developed a series of optical and iodinated tethered Hsp90 inhibitors that have exquisite selectivity *in vivo* for metastatic breast tumors expressing ectopic (cell surface) Hsp90 (Jared J. Barrott, 2013). We also discovered that ectopically expressed Hsp90 is rapidly internalized and can carry these tethered inhibitors specifically into the breast cancer cells. This work in tandem with published clinical results suggests that selective targeting of Hsp90 up regulated in malignancy may present an opportunity to not only discriminate indolent tumors from metastatic disease, but also offer a molecularly targeted radiotherapy approach for body wide tumor ablation with low normal tissue toxicity. Herein, we propose to develop a series of tethered Hsp90 inhibitors capable of selectively delivering radioiodine (^{124}I and ^{131}I) or ^{211}At to malignant tumor cells. We envisage a process in which a patient, after standard of care breast exam, is first evaluated for malignancy vs. indolent disease by positron emission tomography (PET) imaging using ^{124}I-labeled tethered inhibitors. Then, in patients with malignancies detected in high contrast to normal tissues, targeted radiotherapy would be preformed at patient-optimized doses of inhibitor labeled with the β-emitter ^{131}I or the α-emitter ^{211}At. **This is an attractive strategy for breast cancer because the same molecules can be used to not only discriminate indolent disease from metastatic, but also enables selective tumor ablation on a personalized level, potentially mitigating life altering side effects commonly associated with current chemotherapeutics or radiation strategies**.

2. Keywords

Radioiodinated, Tethered Hsp90 inhibitor, malignant breast cancer, stannylation.

3. Accomplishments.

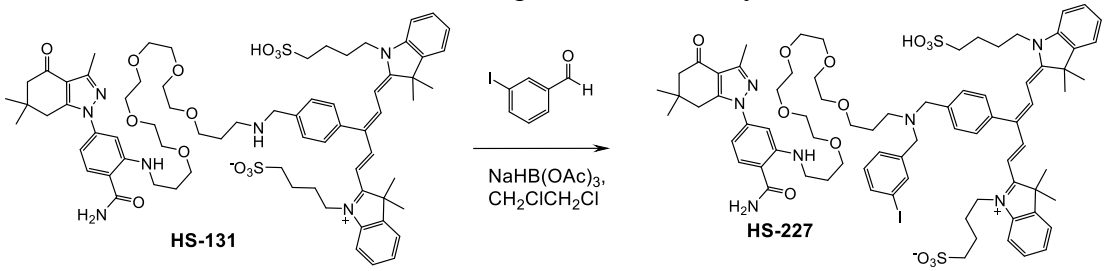
In last year's report, we described the development of a fluorescent PET agent, **HS-131**, where fluorescein was utilized as a fluorescent dye and was introduced using FITC. We developed methodology to prepare the compound with cold iodine and also developed and demonstrated a couple of methods of introducing hot iodine into the compound.



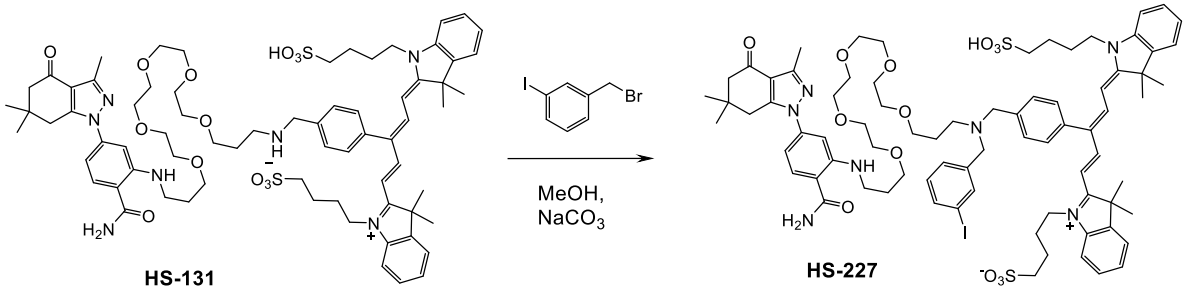
HS-131 R = H Target Fluorescent PET Agent
HS-212 R = CH_3 Inert control molecule

Because of some limitations related to the use of fluorescein, primarily related to background fluorescence in the same spectral region, we became interested in generating similar types of fluorescent compounds that operated at longer wavelengths. In particular, we were interested in making iodinated analogs of some of our near IR probes that utilized cyanine dyes and emitted, depending on the dye, from 680 nm to 820 nm. Detection of these dyes is much more sensitive and much less prone to interference. We have also generated a substantial body of data with these near IR probes.

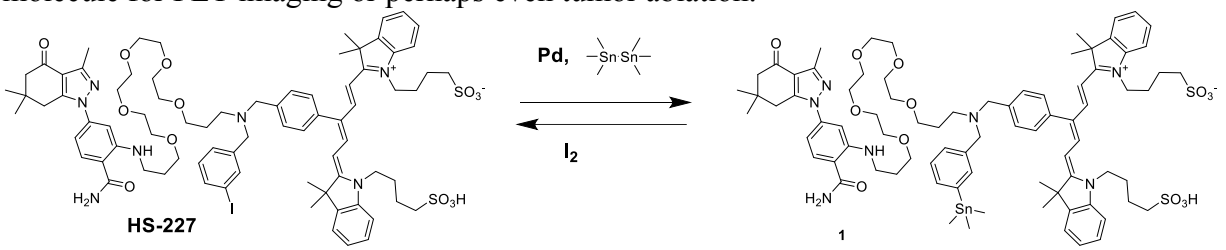
Initially, a number of unsuccessful attempts were made to introduce an aryl iodide onto HS-131. Reductive amination, acylation and alkylation strategies all yielded multiple products in highly colored reaction mixtures. Finally, by repeatedly running a reductive alkylation reaction with sub-stoichiometric amounts of reducing agent followed by repeated chromatography, we were able to isolate about 11% of the desired product, at least by LC/MS.



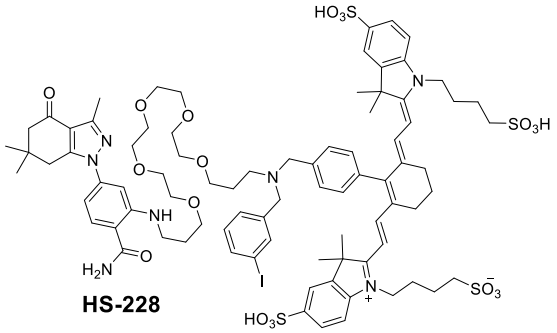
Unfortunately, numerous attempts to increase the yield or even reproduce the reaction were unsuccessful. However, with some of the product in hand as a standard, we returned to the alkylation reaction. By running the reaction in methanol, with solid sodium carbonate added as an insoluble base, the product was smoothly alkylated to give a product matching the product from reductive alkylation. After purification (59% isolated yield) away from some bis-alkylation, LC/MS and NMR confirmed the structure and the compound was registered as HS-227.



With useful quantities of HS-227 in hand, we were able to demonstrate its palladium mediated conversion to a trimethyltin analogue. We were then able to convert the compound back to the iodide. This conversion demonstrates the possibility of introducing other iodine isotopes into the molecule for PET imaging or perhaps even tumor ablation.



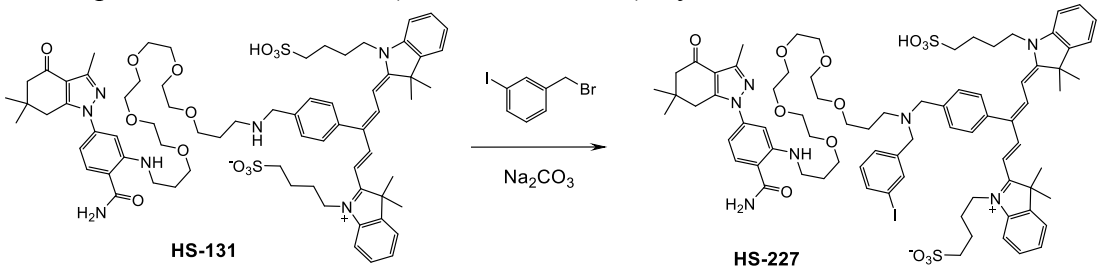
Using the same alkylation methodology, we also made an iodo-analog of HS-196 in good yield.



Further work on the stannylation reaction is needed. A large quantity of proto-destanylation is seen in the reaction. Also, the stannylation needs to be demonstrated on HS-228. Samples of HS-227 and HS-228 were given to the Lylerly lab for imaging analysis. Experimental details are included in the appendix.

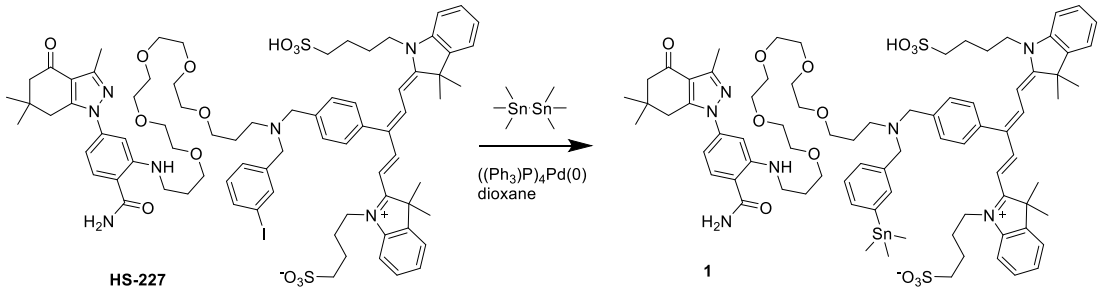
Experimental details.

Reagents were obtained from commercial sources and used without further purification. Proton NMR spectra were obtained on Varian 400 and 500 MHz spectrometers. LC/MS were obtained on an Agilent ion-trap LC/MS system. HRMS results were obtained on an Agilent 6224 LCMS-TOF and are reported as an average of four runs. The syntheses of **HS-131** and compound **3** have been reported in the literature.(Crowe et al., 2017) Dye **2** was obtained from Licor, Inc.

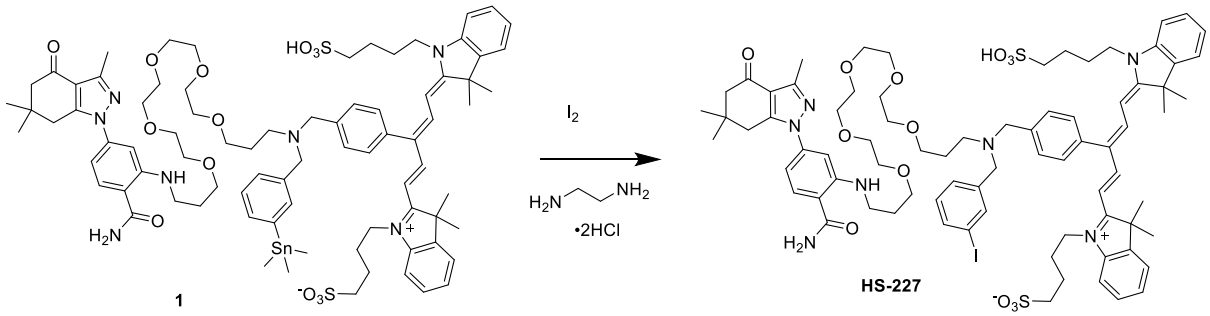


4-(2-((1E,3Z)-3-(4-(21-((2-carbamoyl-5-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1H-indazol-1-yl)phenyl)amino)-2-(3-iodobenzyl)-6,9,12,15,18-pentaoxa-2-azahenicosyl)phenyl)-5-((E)-3,3-dimethyl-1-(4-sulfobutyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-

indol-1-ium-1-yl)butane-1-sulfonate (HS-227). Compound **HS-131** (200 mg, 152 μmol), sodium carbonate (48 mg, 455 μmol) and 3-iodobenzyl bromide (59 mg, 197 μmol) were dissolved in methanol (8 mL) and stirred at RT for 20 h. The reaction mixture was then concentrated and chromatographed twice (150 g C-18, 0 to 100% MeOH with 0.2% formic acid) to give product **HS-227** (136 mg, 59%) as a blue solid. LC/MS shows a single peak with m/z = 767.8 $[M+2H]^{2+}$. ^{1}H-NMR (dmso-d_6) δ 10.32 (br s, 1H), 8.47 (d, J = 14 Hz, 2H), 8.40 (br t, 1H), 8.03 (s, 1H), 7.93 (br s, 1H), 7.73-7.84 (m, 4H) 7.63 (d, J = 7 Hz, 2H), 7.35-7.43 (m, 6H), 7.26 (t, J = 7 Hz, 2H), 7.24 (d, J = 7 Hz, 2H), 6.76 (s, 1H), 6.67 (d, J = 7 Hz, 1H), 5.69 (d, J = 14 Hz, 2H), 4.46 (br m, 2H), 4.38 (br m, 2H), 3.82 (br m, 2H), 3.6-3.72 (m, 4H) 3.38-3.50 (m, 20H), 3.19 (m, 2H), 3.08 (m, 2H), 2.91 (s, 2H), 2.42 (m, 2H), 2.39 (s, 3H), 2.32 (s, 2H), 2.10 (m, 2H), 1.79 (m, 2H), 1.75 (s, 6H), 1.73 (s, 6H), 1.42-1.65 (br m, 8H), 1.00 (s, 6H).



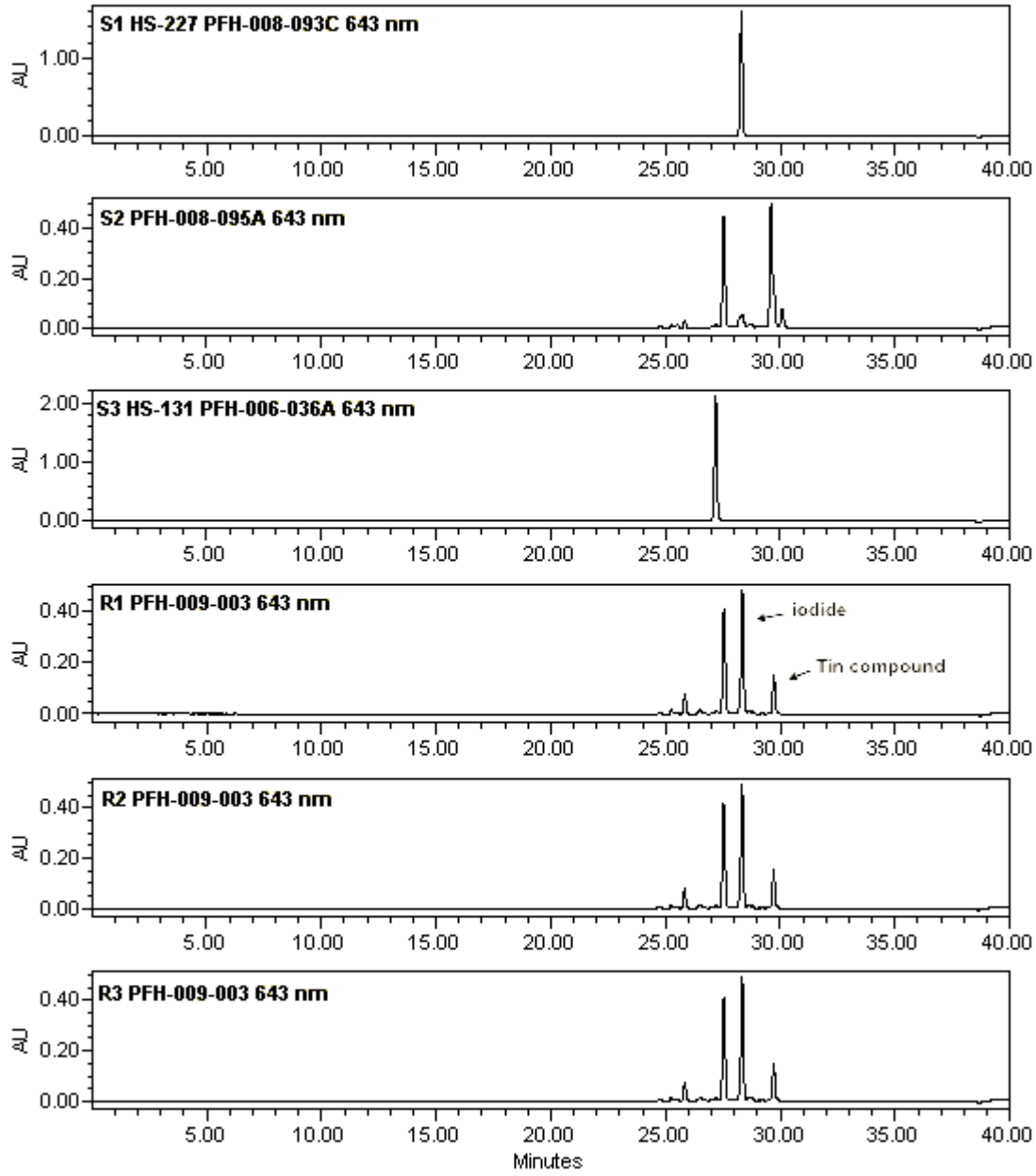
HS-227 (50 mg, 33 μmol), hexamethylditin (14 mg, 9 μL, 42 μmol) and tetrakis triphenylphosphine palladium(0) (1 mg 1 μmol) were slurried in dioxane (1 mL), purged with nitrogen for 30 m and heated to 100 ºC for an hour. The reaction was mixture was adsorbed onto silica gel and chromatographed (50 g C18, 0.2% formic acid to 100% MeOH) to give product **1** (13.9 mg, 27 %) as a blue solid. LC/MS gives a single peak with a little shoulder with m/z = 785.8 $[M+2H]^{2+}$ as part of a cluster typical of Tin compounds. LC/MS at a later date showed significant decomposition, primarily to the hydride.



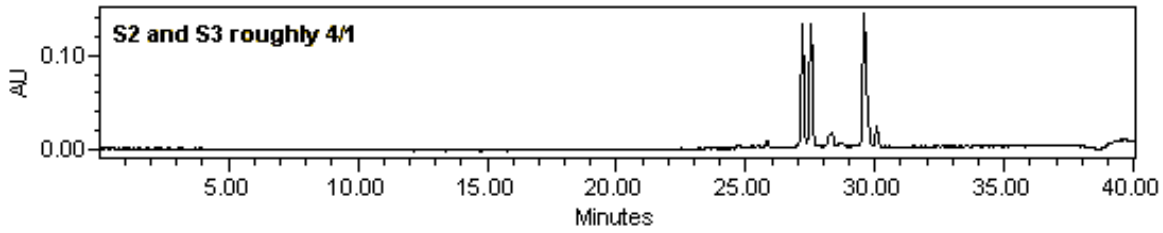
HS-227 and **1** were dissolved in methanol to make 1 mM solutions. A stock 100 mM solution of iodine in methanol was diluted 10-fold to give a 10 mM solution. A stock 1 M solution of ethylene diamine di hydrochloride in water was diluted 10-fold with methanol or water to give a 100 mM solutions. It's complicated but try to follow. 6 samples were prepared.

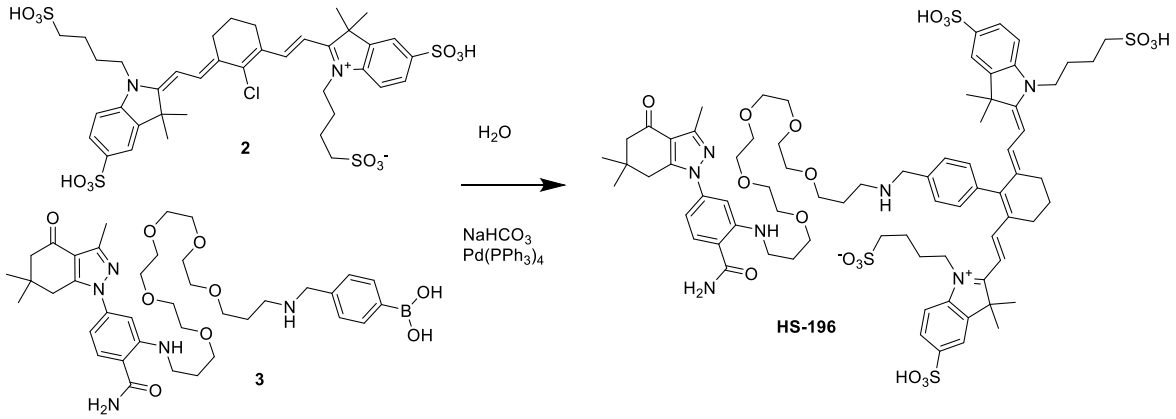
1. S1 50 μL of **HS-227** was diluted 10-fold with methanol. 500 μL tot.
2. S2 50 μL of **1** was diluted 10-fold with methanol. 500 μL tot.
3. S3 500 μL of **HS-131** 100 μM solution in methanol.
4. R1 50 μL of **1** was treated with 5 μL of 100 mM methanolic ethylenediamine dihydrochloride followed by 3 μL of 10 mM iodine solution. The solution was then diluted to 500 μL tot.
5. R2 50 μL of **1** was treated with 5 μL of 100 mM aqueous ethylenediamine dihydrochloride followed by 3 μL of 10 mM iodine solution. The solution was then diluted to 500 μL tot.
6. R3 50 μL of **1** was treated with 5 μL of 100 mM aqueous ethylenediamine dihydrochloride followed by 3 μL of 10 mM iodine solution. This solution was left overnight and diluted to 500 μL the next day.

Samples S1, S2, S3, R1, R2, R3, were analyzed by HPLC (Zorbax Eclipse Plus C18 4.6 x 15 cm 5-micron. 30 minute linear gradient from 100% water with 0.2% formic acid to 100% methanol)

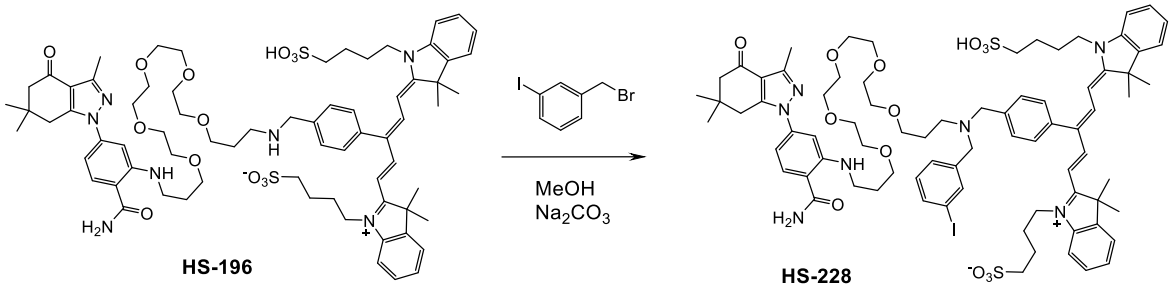


To show that the decomposition of **1** was not debenylation, **1** and **HS-131** were co-injected. Also, LC/MS showed no **HS-131.**





Phenylborate **3** (71 mg, 96 µmol), dye **2**, (85 mg, 95 µmol), tetrakis(triphenylphosphine) - palladium(0) (11 mg, 9.6 µmol) and sodium bicarbonate (46 mg, 431 µmol) were combined in water (2 mL), bubbled with N_2 for 30 m and heated to 100ºC for 1 h. The reaction mixture was dissolved in water and passed through Dowex-50 (2.75 g) onto an Isco 150 g C-18 column and chromatographed (0 to 100% MeOH in water) to give the product **108** (33.3 mg, 22%) as a dark green solid. LC/MS gave a single broad peak with m/z = 772.9 $[M+2]^{2+}$ and m/z = 771.0 $[M-2]^{2-}$. ^{1}H NMR NMR (DMSO-d_6) δ 8.98 (br s, 2H), 7.74 (d, J = 8 Hz, 1H), 7.72 (d, J = 7 Hz, 2H), 7.56 (d, J = 7.0 Hz, 2H), 7.55 (s, 2H), 7.35 (d, J = 8 Hz, 2H), 7.28 (d, J = 8 Hz, 2H), 7.04 (d, J = 14 Hz, 2H), 6.77 (br s, 1H), 6.66 (d, J = 8 Hz, 1H), 6.23 (d, J = 14 Hz, 2H), 4.86 (v br, water), 4.38 (br t, J = 7.0 Hz, 4H), 4.05 (br m, 4H), 3.42-3.57 (m, 20H), 3.19 (t, J = 7.0 Hz, 2H), 3.07 (br m, 2H), 2.91 (s, 2H), 2.69 (br m, 4H), 2.51 (t, J = 7.0 Hz, 4H), 2.49 (DMSO), 2.38 (s, 3H), 2.32 (s, 2H), 2.01 (m, 2H), 1.94 (m, 2H), 1.79 (p, J = 7.0 Hz, 2H), 1.58-1.74 (br m, 8H), 1.12 (s, 12H), 1.00 (s, 6H).



Compound **HS-196** (200 mg, 152 µmol), sodium carbonate (48 mg, 455 µmol) and 3-iodobenzyl bromide (59 mg, 197 µmol) were dissolved in methanol (8 mL) and stirred at RT for 20 h. The reaction mixture was then concentrated and chromatographed twice (150 g C-18, 0 to 100% MeOH with 0.2% formic acid) to give product **228** (136 mg, 59%) as a blue solid. LC/MS shows a single peak with m/z = 767.8 $[M+2H]^{2+}$. ^{1}H-NMR (dmso-d_6) δ 10.32 (br s, 1H), 8.47 (d, J = 14 Hz, 2H), 8.40 (br t, 1H), 8.03 (s, 1H), 7.93 (br s, 1H), 7.73-7.84 (m, 4H) 7.63 (d, J = 7 Hz, 2H), 7.35-7.43 (m, 6H), 7.26 (t, J = 7 Hz, 2H), 7.24 (d, J = 7 Hz, 2H), 6.76 (s, 1H), 6.67 (d, J = 7 Hz,

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4. Impact.

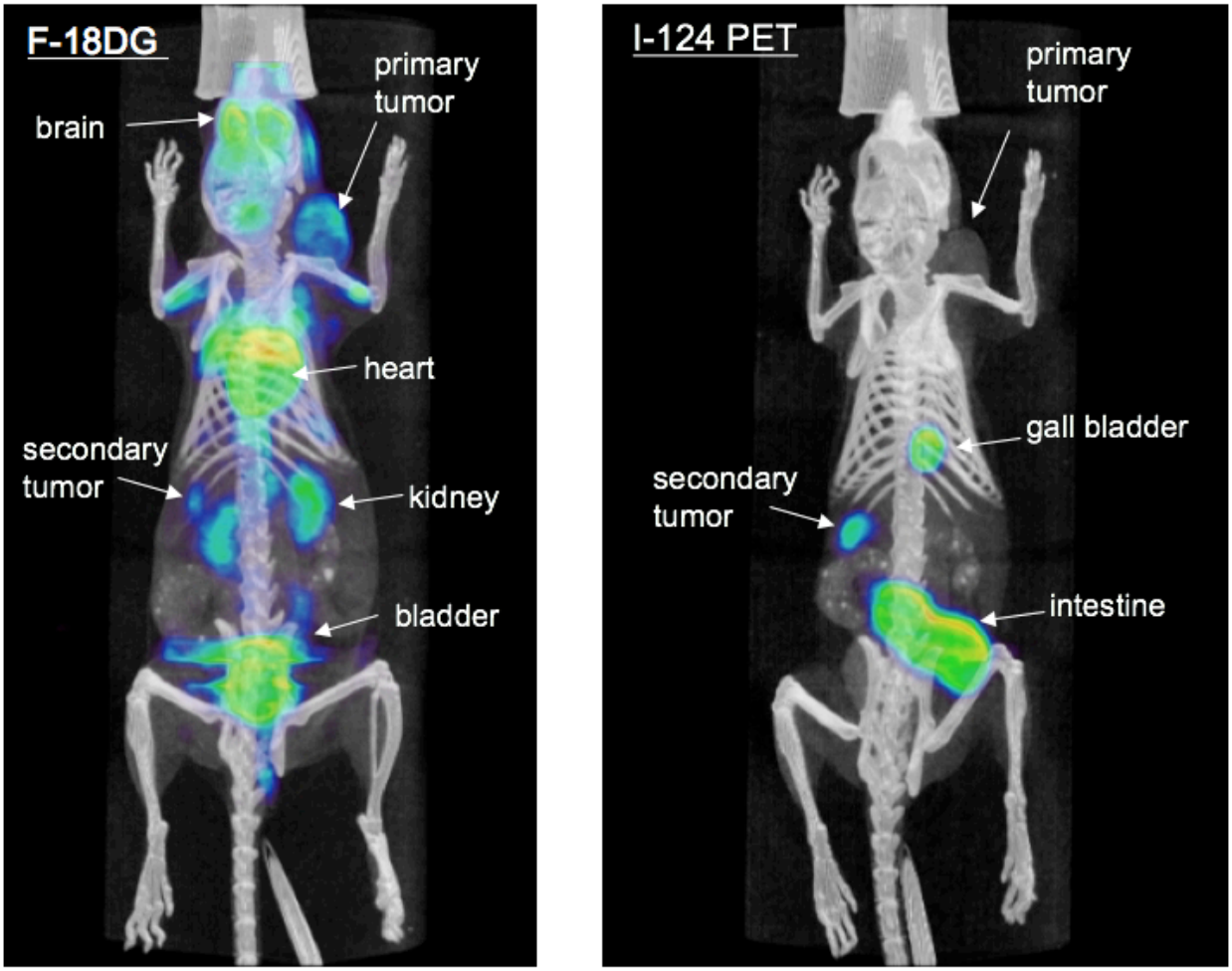

Figure. 1 PET/CT imaging comparing the biodistribution of F18DG with HS-113 a Fluorophore-I^{124} labeled tethered Hsp90 inhibitor. An MMTV-neu mouse was first injected via tail vein catheter with a bolus of F18DG and imaged 60 mins later by PET/CT. 24 hr later the same animal was injected with I^{124}labeled HS-113 (trace and the animal imaged 60 min later by PET/CT. (green = radiotracer signal; grey = CT signal).

In collaboration with the Small Animal Imaging (SAI) core and mouse phase 1 unit (MP1U) at UNC we compared the biodistribution of HS113 to ^{18}F deoxyglucose (^{18}FDG) in an MMTV-Neu mouse model of HER2+ breast cancer (Fig.1). To obtain the PET/CT images shown in figure 3, ^{18}FDG was injected 24 hours prior to HS-113. Figure 1 shows that ^{18}FDG has broad biodistribution throughout the animal by 60 mins and is found in several major organs, brain, eye, heart, lymph nodes kidney, liver and bladder. Uptake` is also detected in two tumors, a large primary tumor mass at the neck region and on the left flank below the ribs. In contrast, at 60 min HS113 uptake is observed only in the gall bladder, lower bowel and the small secondary tumor on the left flank. The lack of uptake by HS113 of the primary tumor may reflect its necrotic state compared to the more actively growing secondary tumor. One of the most striking feature of HS113, is its rapid and exclusive clearance through the bile in comparison to all other fluorophore-tethered Hsp90 inhibitors we have examined in tumor baring animals. Without exception elimination primarily occurs through the kidneys and urine. Whole body real time PET imaging over the first 30 min reveals the extent to which ^{124}IHS-113 is cleared by the hepatobiliary system (Fig.2). In the time lapse sequence, after 10 min the probe starts to discriminate the outlines of the liver and by 15-20 minutes the architecture of biliary ducts are revealed, until the majority of the probe collects as an intense signal in the gall bladder followed

by delivery as a large bolus to the intestine. These findings suggest that addition of the benzyl iodide alone dramatically alters the rate and route of probe clearance compared to prior fluorophore-tethered inhibitors. Two explanations may explain this phenomenon, either the molecule is rapidly metabolized releasing the iodinated prosthetic or HS-113 interacts in some manner with the hepatobiliary next work via the benzyl iodide group.

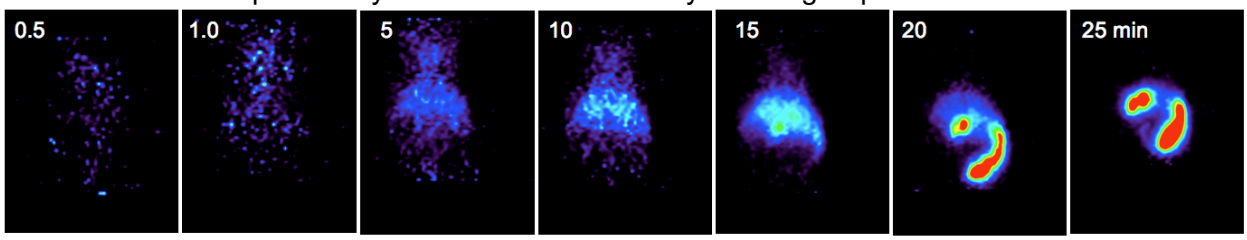

Figure 2. Time lapse sequence by PET following the elimination of I^{124}HS-113 in a mouse. The animal was injected (IV) with a tracer amount of I^{124}HS113 and the signal followed continuously over 30 min. Images were captured from a movie following the tracer's biodistribution.

5. Changes/Problems.

With any chemistry program one can anticipate encountering technical issues during the synthesis of a molecule. Major issues include poor yield, loss of solubility, unpredicted product formation, difficulties with selective iodination or stannylation. Compounds that exhibit such behavior will not be pursued. One anticipated issue is the impact of the imaging moiety and its effect on cellular internalized. Although we would still classify our tethered inhibitors as small molecules (average MW 800-1400 Da) they do fall outside of classically held rules of druggability such as Lipinski's rule of 5 (Lipinski, 2000). Indeed, generally our molecules are polar and have low cLogP values compared to PU-H71 and SNX5422, preventing them freely diffusing into cells. In instances where the imaging moiety has a large impact on internalization we will replace this with other fluorophore or iodine carrying functionality. As discussed, we have observed that choice of these moieties dramatically effects the PK properties and clearance time ($T_{1/2}$) of our tethered inhibitors from the tumor, if not their selectivity for Hsp90. Generally larger more polar moieties (e.g. fluorescein compared with NrIR versions slow the rate of elimination, whereas addition of smaller molecules such as a benzyl group increases uptake. In many ways defining which iodine or fluor carrying moiety is chosen will be an empirically driven process. We anticipate that our studies with cell lines will address such issues in advance of animal studies. However, we recognize that even the best performers in cell-based studies will behave unpredictably in animal. We believe on the imaging side there is sufficient diversity of commercially available fluorophors of varying structural diversity to enable us to define one or more tethered inhibitors that will meet our criteria. As discussed, an idealized tethered inhibitor will carry both radioiodine and a fluorophore, the latter enabling tumor cell selectivity within a biopsy to be determined by confocal microscopy. We have successfully synthesized at least one duel-imaging molecule, HS113, carrying fluorescein and iodine. In some respects, the fluorophores can be seen as surrogate for adding chemical bulk and diversity to our molecules for altering PK characteristics, with the added advantage of being fluorescent.

We are somewhat limited in the diversity of the types of molecules that can carry iodine stably. Our current lead iodine carrying molecule is a benzylamine e.g. HS-113. The aryl-iodine in this molecule is predicted to be highly stable *in vivo* and unlikely to leach the incorporated iodine into the body where it could be taken up into the thyroid. Other obvious choices that carry iodine are not considered ideal, e.g. phenols and amino acids such as tyrosine (found in thyroxine). These are also less stable and likely to be metabolized in non-tumor tissues if not directly transported into the thyroid. However, if our benzylamide versions fail in animal studies because of rapid clearance from the circulation, phenols and amino acids such as tyrosine or phenylalanine offer alternate approaches. Another strategy for altering PK involves switching the ligand portion of the molecules to other Hsp90 inhibitors that may have higher affinity for Hsp90 or different PK properties than the HS-10 indoline scaffold e.g. Ganestespib and NVP-AUY922 (Eccles et al., 2008; Shimamura et al., 2012). If it becomes intractable to use iodine as a radio-label we will explore the use of ^{18}F. This is a high-energy emitter with a short half-life

(~12 mins) compared with various radioisotopes of iodine which can range from a few days (^{124}I) to weeks (^{121}I). There are several strategies for incorporating ^{18}F, the most straightforward would be to use the methods of Vasdev (Rotstein et al., 2016), whereby iodine is directly activated for fluoride displacement. Another alternative would be to utilize one of several BODIPY dyes for imaging and use the methods of Li (Liu et al., 2014a) or Mazitschek (Hendricks et al., 2012) for ^{18}F displacement on the dye. A third approach would involve using an alternative labeling moiety based on a fluoride substitution or exchange on a boronate as developed in the Perrin lab(Liu et al., 2014b; Liu et al., 2014c).

6. Products

Several novel radio-iodinated tethered Hsp90 inhibitors have been developed carrying iodine and succsefully labeld with I^{124} . HS113 show promise as a novel PET imnaging agent in mouse models of human breast cancer.

7. Participants & Other Collaborating Organizations……

Grants.gov ID Number	GRANT11490422
Principal Investigator	Michael Zalutsky
Performing Organization	Duke University
Contracting Organization	Duke University
Partner Budget Requested	$549,500
Direct Costs	$350,000
Indirect Costs	$199,500

8. Special Reporting Requirements

none

9. Appendices

References:

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